Exhibit 1

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ΔN-p53, a natural isoform of p53 lacking the first transactivation domain, counteracts growth suppression by wild-type p53

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The tumor suppressor protein p53 is ubiquitously expressed as a major isoform of 53 kD, but several forms of lower molecular weight have been observed. Here, we describe a new isoform, ΔN-p53, produced by internal initiation of translation at codon 40 and lacking the N-terminal first transactivation domain. This isoform has impaired transcriptional activation capacity, and does not complex with the p53 regulatory protein Mdm2. Furthermore, ΔN-p53 oligomerizes with full-length p53 (FL-p53) and negatively regulates its transcriptional and growth-suppressive activities. Consistent with the lack of Mdm2 binding, ΔN-p53 does not accumulate in response to DNA-damage, suggesting that this isoform is not involved in the response to genotoxic stress. However, in serum-starved cells expressing wild-type p53, ΔN-p53 becomes the predominant p53 form during the synchronous progression into S phase after serum stimulation. These results suggest that ΔN-p53 may play a role as a transient, negative regulator of p53 during cell cycle progression.
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Introduction

TP53 belongs to a family that also includes *TP73* and *TP63*. The products of these genes share a common transcription factor architecture, with a N-terminal transactivation domain, a central hydrophobic domain with sequence-specific DNA-binding activity and a highly variable C-terminus, with oligomerization and regulatory domains. *TP63* (chromosome 3q27-29) and *TP73* (chromosome 1p36-33) are expressed in a differentiation-related manner in a wide range of fetal and adult tissues (Levrero *et al.*, 2000; Yang *et al.*, 2002). While p63 plays an essential role in epidermal-mesenchymal interactions during embryonic development and is critical for maintaining the progenitor population in epithelial tissues, p73 is implicated in neurogenesis, sensory pathways as well as homeostatic stimuli control (Mills *et al.*, 1999; Yang *et al.*, 1999, 2000). *TP73* and *TP63* are both expressed as multiple isoforms, resulting from a combination of alternative splicing events, affecting both N- and C-terminal parts of the protein. ΔN-isoforms, which lack the minimal transactivation domain in the N-terminus, are unable to up-regulate the expression of target genes, but interact with full-length isoforms to down-regulate their activity in a dominant manner (Levrero *et al.*, 2000; Pozniak *et al.*, 2000; Yang *et al.*, 2000). Studies on p63 and p73 expression in squamous epithelia indicate that a spatial and temporal switch in isoform expression participates in the maintenance of the normal differentiation pattern (Nylander *et al.*, 2000).

TP53 differs from other family members by its ubiquitous expression, its post-translational inducibility in response to multiple forms of stress and its expression as a single, major isoform of 53 kDa (Hainaut and Hollstein, 2000; Wahl and Carr, 2001). The main function of *TP53* is tumour suppression, through its capacity to act as a sequence-specific transcriptional regulator and to bind to several classes of proteins. These multiple functions cooperate to down-regulate cell cycle progression, to induce apoptosis, to enhance DNA repair and to stimulate differentiation (Balint and Vousden, 2001; Bargonetti and Manfredi, 2002; Hainaut and Hollstein, 2000; Wahl and Carr, 2001). Genes expressed in response to p53 activation include regulators of cell cycle (*$P21^{WAF1}$*, *CYCLIN G*, *GADD45*), or apoptosis (*BAX*, *p53AIP1*, *PUMA*, *PIG3*) (Bargonetti and Manfredi, 2002). The induction and stability of p53 are controlled by Mdm2, which binds p53 in the N-terminus and targets it for proteosome-mediated degradation. The Mdm2 gene is transcriptionally

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activated by p53, thus defining an auto-regulatory feedback loop between the two proteins (Michael and Oren, 2002).

In cancers, *TP53* functions are frequently inactivated by missense mutations, most of which alter the DNA-binding domain (Hainaut and Hollstein, 2000). There is evidence that several common mutations associated with cancers not only inactivate DNA-binding, but also confer to the protein a dominant, oncogenic activity (Dittmer *et al.*, 1993). Recently, it has been proposed that dominant activity may result at least in part from the specific binding and inactivation of p63 or p73 isoforms by mutant p53 (Strano *et al.*, 2000).

Here we describe an isoform of p53, ΔN-p53, lacking the N-terminus. This natural variant is expressed by internal initiation of translation at an AUG codon at position 40 and is not inducible in response to stress. ΔN-p53 is detectable in normal and cancer cell lines as well as in normal human tissues. ΔN-p53 interacts with wild type p53 and down-regulates its suppressor activity. In addition, the ratio between the levels of FL-p53 and ΔN-p53 varies during cell cycle, raising the possibility that ΔN-p53 plays a role in the control of progression into entry in the S phase.

Results and Discussion

We first detected the ΔN-p53 isoform in the breast cancer cell line 21PT, which is unable to synthesize full-length p53 (FL-p53) due to an insertion at codon 33 but contains an intact *TP53* coding sequence downstream of codon 33 (Liu *et al.*, 1994). This cell line was considered as p53-null since no p53 protein could be detected by Western blot with the monoclonal antibody BP53.12, which recognizes residues 20–25 (Liu *et al.*, 1994). However, we were able to detect a ΔN-isoform by gel shift assay and by Western blot using PAb1801, recognizing an epitope located downstream of codon 33 (residues 46–55). Figure 1a compares DNA-binding activities in MCF-7 and 21PT cells exposed to 150 μM hydrogen peroxide as a DNA-damaging agent. In MCF-7 cells, peroxide induced a rapid and transient increase in DNA-binding, with a maximum after 4 h and a decrease to basal levels after 12 h. In 21PT, a basal DNA-binding activity was detected, which was decreased after exposure to peroxide but returned to basal levels after 12 h. No reactivity was detected in the absence of the antibody PAb421, which stabilizes (and super-shifts) complexes between p53 and specific oligonucleotides *in vitro* (data not shown). Supershift experiments with antibodies DO7 (residues 21–25) (Stephen *et al.*, 1995) and PAb1801 (residues 46–55) (Legros *et al.*, 1994) showed that only the latter supershifted the complexes in 21PT cells (Figure 1b). This result indicates that 21PT cells express a form of p53 which does not react with DO7, is not inducible by peroxide but retains the capacity to bind to a p53-consensus DNA sequence. To verify that the clone of 21PT we used had not diverged from the one described by Liu *et al.* (1994), sequencing was

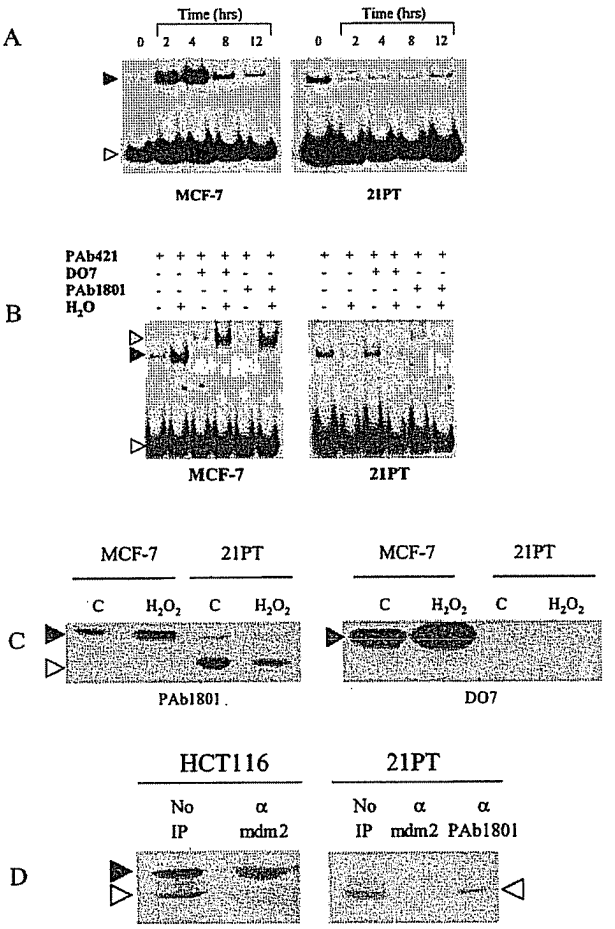

Figure 1 Identification of ΔN-p53. (a) Detection of a p53 DNA-binding activity by Electromobility Shift Assay as described in Verhaegh *et al.* (1997). MCR-7 cells (control expressing FL-p53) and 21PT were exposed to 150 μM hydrogen peroxide (for 2–12 h) to induce p53. Aliquots of nuclear extracts were incubated with a ^{32}P-labeled synthetic p53-consensus DNA-binding sequence ($p53^{CON}$, 5′-GGACATGCCCGGGCATGTCC-3′) in the presence of PAb421, which binds to the C-terminus of p53 and stabilizes p53:DNA interactions. Only a portion of the autoradiogram is shown. Black arrow: p53:PAb421:DNA complexes. The white arrow corresponds to an unknown protein that binds DNA in a non-sequence specific manner. (b) Supershift experiment using antibodies against p53 N-terminus. The mobility of p53:PAb421:DNA complexes (black arrow) was tested in the presence of monoclonal antibodies DO7 and PAb1801, recognizing different epitopes in the N-terminal region of p53 (DO7: residues 19–26, Dako; PAb1801: 46–55, Oncogene Research Products). In MCF-7, both antibodies induce a super-shift of the complex (grey arrow). In 21PT, a super-shift of the protein-DNA complex was only detected in presence of PAb1801. (c) Presence of a 40 kDa isoform of p53 in 21PT. MCF-7 or 21PT cells were exposed for 2 h to peroxide at 150 μM, to induce p53 accumulation and activation. Extracts of unexposed (C, Control) and exposed (H_2O_2) cells were analysed by Western blot with PAb1801 (left panel) and DO7 (right panel). (d) Absence of interaction between Mdm2 and ΔN-p53. Mdm2/p53 complexes were investigated in HCT116 cells (expressing two isoforms of wild-type p53, FL-p53 and ΔN-p53) and in 21PT (expressing ΔN-p53). Cell extracts were either directly analysed by Western blot with CM1 (purified rabbit IgG to p53, Novocastra) or immunoprecipitated with the anti-Mdm2 antibody (Calbiochem), followed by Western blotting of the precipitates with CM1. Black arrow: FL-p53; white arrow: ΔN-p53

performed to confirm the +1 bp insertion at codon 33 (data not shown). Comparative Western blot analysis indicated that 21PT cells contained a 40 kDa form of p53, reactive with PAb1801 but not with DO7 (Figure 1c). In addition, co-immunoprecipitation studies revealed that this 40 kDa form did not complex with Mdm2 (Figure 1d). Together, these results indicate that 21PT cells expressed a form of p53 that lacks the N-terminus. A protein with similar electrophoretic mobility and immunoreactivity was detectable in several cell lines expressing wild type p53 (HCT116, A549, HepG2, WI-38, data not shown).

In mouse fibroblasts transfected with full-length human *TP53* cDNA, two bands were clearly detected, corresponding to FL-p53 at 53 kDa and ΔN-p53 at 40 kDa, respectively, indicating that the two proteins were expressed from a single mRNA (Figure 2a). Mutation of an in-frame AUG at codon 40 resulted in the disappearance of ΔN-p53, but not of FL-p53 (Figure 2b). RT–PCR analysis of p53 expression using different sets of primers in exons 1–4 did not reveal the presence of alternatively spliced forms of mRNA in HCT116 cells, despite the presence of ΔN-p53 protein (data not shown). These results clearly point to alternative initiation of translation at codon 40 as the main mechanism for generation of ΔN-p53.

Codon 40 (position 118) lies within a well-defined sequence environment matching Kozak's consensus criteria (Figure 2c) (Kozak, 1987). This codon and its Kozak environment, are conserved in many species including human, rat, mouse, woodchuck and chick. However, this sequence is not present in the *TP53* gene of cow, xenopus, rhesus, horse and pig, suggesting that expression of ΔN-p53 may be restricted to some species. No mutations at codon 40 have been reported in human cancers (IARC *TP53* database, 2002 update, 16231 mutations).

Variant forms of p53 lacking the N-terminus have been previously reported but were interpreted as resulting from proteolytic cleavage of FL-p53. First, Kubbutat and Vousden (1997) have shown the existence of a calpain-sensitive site at residues 13–19. Calpain digestion *in vitro* resulted in the production of a 46 kDa protein, larger than the isoform described here. Second, auto-proteolytic cleavage p53 products of 50, 40, 35 and 30 kDa were described after incubation of wild-type 53 with single stranded or damaged DNA (Molinari *et al.*, 1996; Okorokov and Milner, 1997). The 40 kDa product was generated by N-terminal cleavage of the protein. However, the existence of this fragment was not documented in intact cells. We believe that the ΔN-p53 identified here differs from this auto-proteolytic cleavage product. First, ΔN-p53 is detectable in the absence of DNA-damage, whereas auto-proteolytic cleavage has been reported to depend upon binding to damaged DNA. Second, our results clearly show that expression of ΔN-p53 requires an intact AUG at codon 40.

The production of N-terminally deleted isoforms by internal initiation of translation is a common feature in several transcription factors including VHL, WT1 and human estrogen receptor (Barraille *et al.*, 1999; Blankenship *et al.*, 1999; Scharnhorst *et al.*, 1999). In Mdm2, Veldhoen *et al.* (1999) have described a complex mechanism of regulation of expression

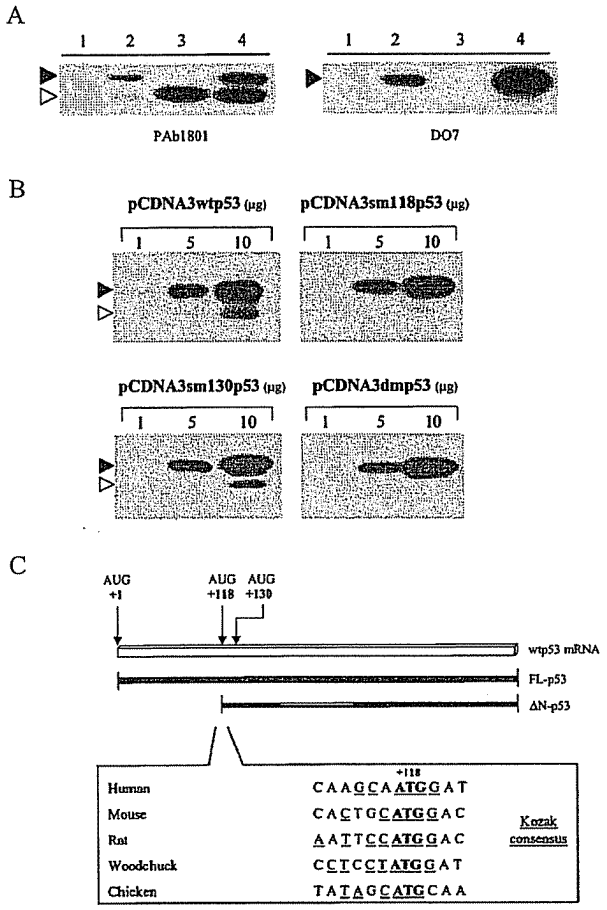

Figure 2 ΔN-p53 is synthesized by internal initiation of translation at codon 40. (a) Expression of a 40 kDa isoform in p53-null mouse fibroblasts transfected with wild type p53. Balb.c 10.1 fibroblasts were transfected with either FL-p53 cDNA, ΔN-p53 cDNA, or both. All vectors were constructed in pCDNA3. After 24 h, cells were extracted and analysed by Western blotting with PAb1801 or DO7. 1: non-transfected cells; 2: FL-p53; 3: ΔN-p53; 4: FL-p53+ΔN-p53, 1:1. (b) Site-directed mutagenesis of candidates AUG at positions 118 and 130. Site-directed mutagenesis was performed on FL-p53 cDNA using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene). Primers were GCCGTCCCAAGCATTGGATGATTTGATG/CATCAAATCATCCAATGCTTGGGACGGC (position 118); CAATGGATGATTTGTTGCTGTCCCCGGAC/GTCCGGGGACAGCAACAAATCATCCATTG (position 130); AAGCATTGGATGATTTGTTGCTGTC/ACAGCAACAAATCATCCAATGCTTG (double mutant). Extracts of Balb/c 10.1 cells transfected with the site-directed plasmids were analysed by Western blot using PAb1801. pCDNA3wtp53: FL-p53; pCDNA3sm118p53: single missense mutation at position +118; pCDNA3sm130p53: single missense at position +130; pCDNA3dmp53: double missense mutations at positions +118 and +130. Black arrow: FL-p53; white arrow: ΔN-p53. (c) Conservation of Kozak's environment at the site of initiation of ΔN-p53. In-phase AUG codons +118 (codon 40) and +130 (codon 44) are shown. The conservation of the Zozak's environment of AUG at position 118 is shown in various species

involving alternative splicing and initiation at an internal AUG, resulting in the production of an isoform lacking the N-terminal, p53-binding domain (Veldhoen *et al.*, 1999). This Mdm2 isoform may be viewed as a counterpart of ΔN-p53, since the latter lacks the Mdm2-binding domain.

Consistent with the lack of the main element of transcriptional activation domain (residues 1–42), ΔN-p53 did not activate reporter genes containing p53 binding sites derived from the promoters of several p53 target genes, even when expressed at high levels in Balb/c 10.1 cells (Figure 3a,b). One exception was the $P21^{WAF1}$ promoter, which was activated with ΔN-p53 to a level of about 50% of that of FL-p53. However, this effect was not consistent when ΔN-p53 plasmid was transfected at lower concentrations (down to 0.5 ng/dish) and no effect on the endogenous $P21^{WAF1}$ protein was seen (data not shown). Thus, this effect is unlikely to reflect a physiological activation of the $P21^{WAF1}$ promoter. A second transcriptional activation domain has been mapped between 43–63 in p53 (Candau *et al.*, 1997; Zhu *et al.*, 1998). Downstream of this domain lies a proline-rich domain (residues 60–90). Both of these domains are conserved in ΔN-p53 and have been shown to play complementary roles in the transcriptional activation of a subset of p53 target genes involved in apoptosis (Baptiste *et al.*, 2002; Walker and Levine, 1996). Recent evidence indicates that a p53 with mutations at positions 22 and 23, which largely inactivate the main transactivation domain, retains the capacity to induce p53-dependent apoptosis in response to several cytotoxic drugs (Baptiste *et al.*, 2002). Thus, it is possible that ΔN-p53 may have an intrinsic capacity to regulate some aspects of p53-dependent apoptosis when overexpressed in p53-deficient cells. However, our results using reporter constructs do not provide evidence for an autonomous transcriptional activity of ΔN-p53.

ΔN-p53 contains an intact oligomerization domain. Co-immunoprecipitation in murine fibroblasts transfected with both FL-p53 and ΔN-p53 (Figure 3c) and in cells expressing synchronously both forms of the protein (HepG2, Figure 3d) showed that ΔN-p53 could complex with FL-p53. Furthermore, ΔN-p53 down-regulated transcriptional activity of FL-p53 towards a reporter gene (Figure 3e). These effects suggest that excess ΔN-p53 could compete with FL-p53 for binding to specific DNA targets and neutralize the transactivation activity of the oligomeric complex. Consistent with these effects, clonogenic assays showed that ΔN-p53 did not suppress the growth of the p53-null cell line H358, but counteracted the suppressive effect of FL-p53 when present in 10-fold excess (Figure 3f). These results are in agreement with those of Unger *et al.* (1993), who reported that an excess of a deleted p53 protein lacking the N-terminal 43 residues was impaired in transformation suppression activity. Thus, ΔN-p53 may behave as a suppressor of FL-p53 activity.

In order to determine in which conditions ΔN-p53 was expressed, we exposed cell lines expressing wild-type p53 (MCF-7, HCT116 and A549) to various DNA-damaging stimuli including peroxide, UVB, methylmethane sulphonate and cisplatin. In all cases, the ΔN-p53 isoform did not appear to accumulate or to be activated in response to such stresses (data not shown). These observations are consistent with the data in Figure 1, which shows that the DNA binding activity of ΔN-p53 in 21PT cells was decreased after exposure to peroxide. Absence of inducibility in response to DNA damage is compatible with lack of Mdm2 binding. As Mdm2 is considered as the main regulator of p53 protein turnover (Michael and Oren, 2002), the lack of binding to Mdm2 may explain why the highest ratios between levels of ΔN-p53 and FL-p53 were detected in non-stressed, cultured cells (see for example Figure 1d).

We next analysed whether ΔN-p53 levels were affected in other physiological processes where p53 might play a role. Several studies have suggested that p53 activity may represent a limiting factor for cell-cycle progression into S phase. Different mechanisms, including down-regulation of p53 expression in response to c-Jun (Schreiber *et al.*, 1999) and transient conformational inactivation of wild-type p53 (Milner, 1991), have been proposed to explain how p53 protein activity could be specifically down-regulated at the G1-S transition. In Figure 4, we have used the WI-38 human diploid fibroblasts to investigate changes in ΔN-p53 expression during the progression into S phase after cell cycle synchronization by serum-starvation. Western blot analysis showed that progression into cell cycle (Figure 4a) was accompanied by changes in the ratio between levels of FL-p53 and ΔN-p53 (Figure 4b). Both forms were detectable by PAb1801 and CM1 in serum-deprived, arrested cells, whereas only FL-p53 was detected by DO7. After serum restimulation, FL-p53 levels decreased whereas levels of ΔN-p53 increased, the latter form becoming predominant at 24–30 h after serum stimulation. This timing corresponded to the onset of cyclin A expression, which characterizes entry into S phase, and to a drop in $P21^{WAF1}$ expression (Figure 4b), suggesting a transient inactivation of FL-p53 functions. Interestingly, the reactivities of FL-p53 with DO7 and PAb1801 exhibited different patterns of variation with time, indicating that the two antibodies reacted differentially with various sub-populations of FL-p53. It should be noted that the transient decrease in FL-p53 levels observed with DO7 was also observed with CM1. The same fluctuations in FL-p53/ΔN-p53 ratio were observed after re-stimulation of serum-starved HCT116 cells (data not shown). In these cells, however, the re-entry into cell cycle was more rapid than in WI-38 cells, and the increase of ΔN-p53 was detected as early as 4–8 h after serum restimulation. These results are compatible with the hypothesis that ΔN-p53 may act as a 'facilitator' of G1-S transition through its capacity to negatively regulate FL-p53. This effect could be additive to the down-regulation of wild-type p53 expression induced by c-Jun (Schreiber *et al.*, 1999). Further experiments are needed to determine

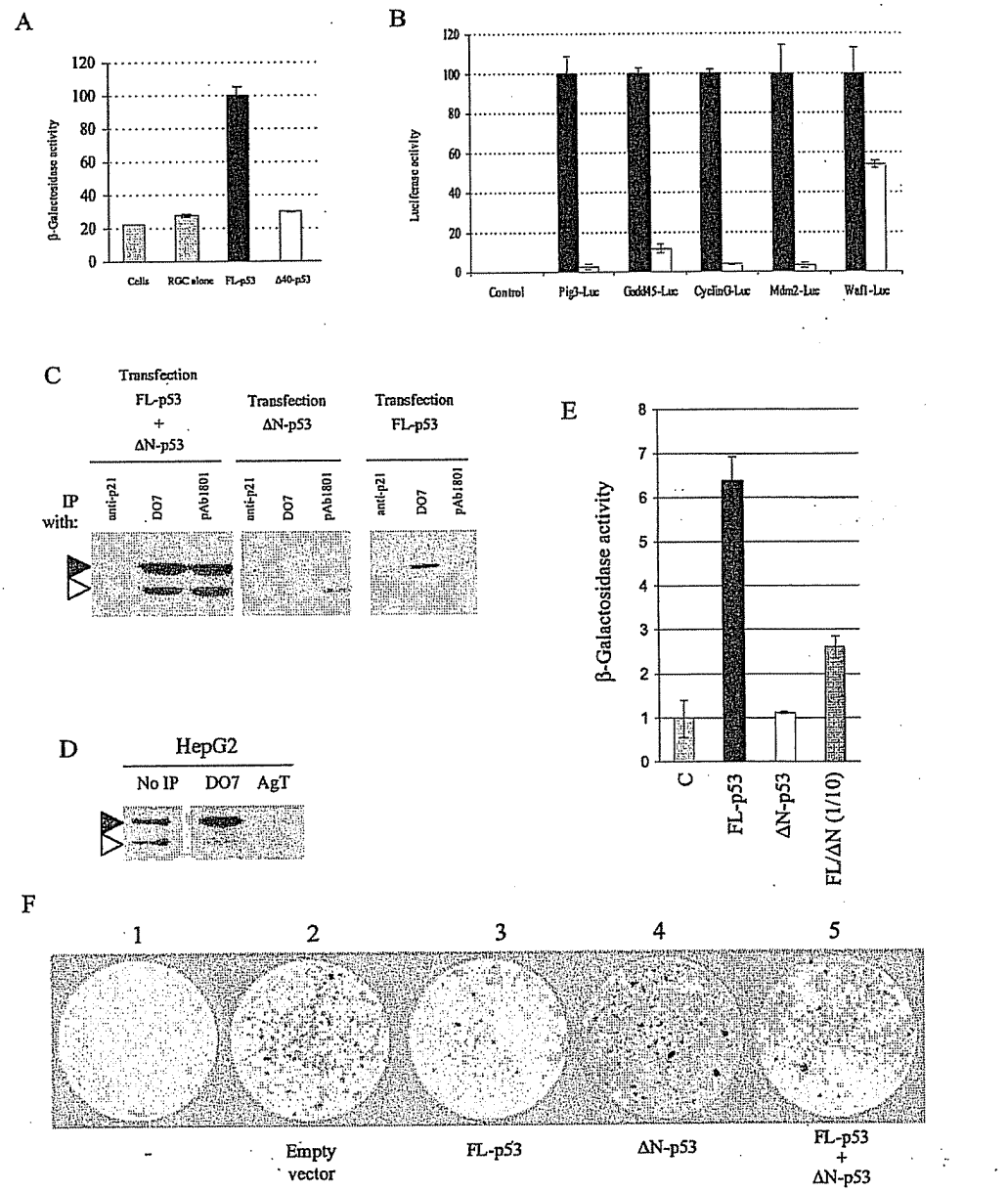

Figure 3 ΔN-p53 lacks transcriptional and growth suppressive activities. (a) Transcriptional activity of ΔN-p53 (white bar) was analysed in Balb/c 10.1 fibroblasts transfected with pCDNA3 vectors as indicated. The pRGCΔFosLacZ plasmid, containing the RGC (Ribosomal Gene Cluster) p53 binding site upstream of the β-galactosidase, was used as a reporter gene. Each bar is the average of three independent experiments. (b) Same experiment as in (a) using luciferase reporter genes containing p53-binding segments of PIG3, GADD45, CYCLIN-G, MDM2 and $P21^{WAF1}$ promoters. Luciferase assays were performed using the Dual-Luciferase™ Reporter Assay System (Promega). White bars: ΔN-p53; black bars: FL-p53. (c) Complex formation between ΔN-p53 and FL-p53. Balb/c 10.1 fibroblasts were transfected with FL-p53, ΔN-p53 or both. Co-immunoprecipitation was performed with DO7, PAb1801 and with an irrelevant antibody (anti-$P21^{WAF1}$, Oncogene Science). Immunoprecipitated proteins were denaturated and analysed by Western blot using the polyclonal antibody CM1. Presence of ΔN-p53 in the DO7 immunoprecipitates, when both FL-p53 and ΔN-p53 were co-expressed, indicates that the two isoforms can interact. Black arrow: FL-p53; white arrow: ΔN-p53. (d) Complex formation between FL-p53 and ΔN-p53 in HepG2 cells, expressing a wild-type p53 as well as the ΔN-p53 isoform (No IP lane). Co-immunoprecipitation was performed with DO7 and an irrelevant antibody (anti-SV40 AgT, Calbiochem). Denaturated immunoprecipitated proteins were analysed by Western blot using the polyclonal antibody CM1. Black arrow: FL-p53; white arrow ΔN-p53. (e) Inhibition of the transcriptional activity of FL-p53 by excess of ΔN-p53. The transactivation activity of co-transfected FL-p53 and ΔN-p53 towards the RGC-consensus sequence was measured. Balb/c 10.1 mouse fibroblasts were transfected with either FL-p53 vector (1 μg; black bars), ΔN-p53 vector (1 μg, white bars) or both vectors (ΔN-p53, 10 μg; FL-p53, 1 μg, dark grey bars). Each bar is the average ± standard deviation of at least three experiments. (f) ΔN-p53 counteracts the growth suppressive activity of FL-p53. Colony formation assay in human, p53 null H358 lung cancer cells. Cells were transfected with 1 μg of either plasmid (pCDNA3, pCDNA3FL-p53, pCDNA3ΔN-p53; wells 2–4). In well 5, FL-p53 and ΔN-p53 were co-transfected at a plasmid ratio 1 : 10. Well 1: non transfected cells. After transfection, cells were selected with G418 (800 μg/ml) for 2–3 weeks, fixed and stained with Giemsa stain

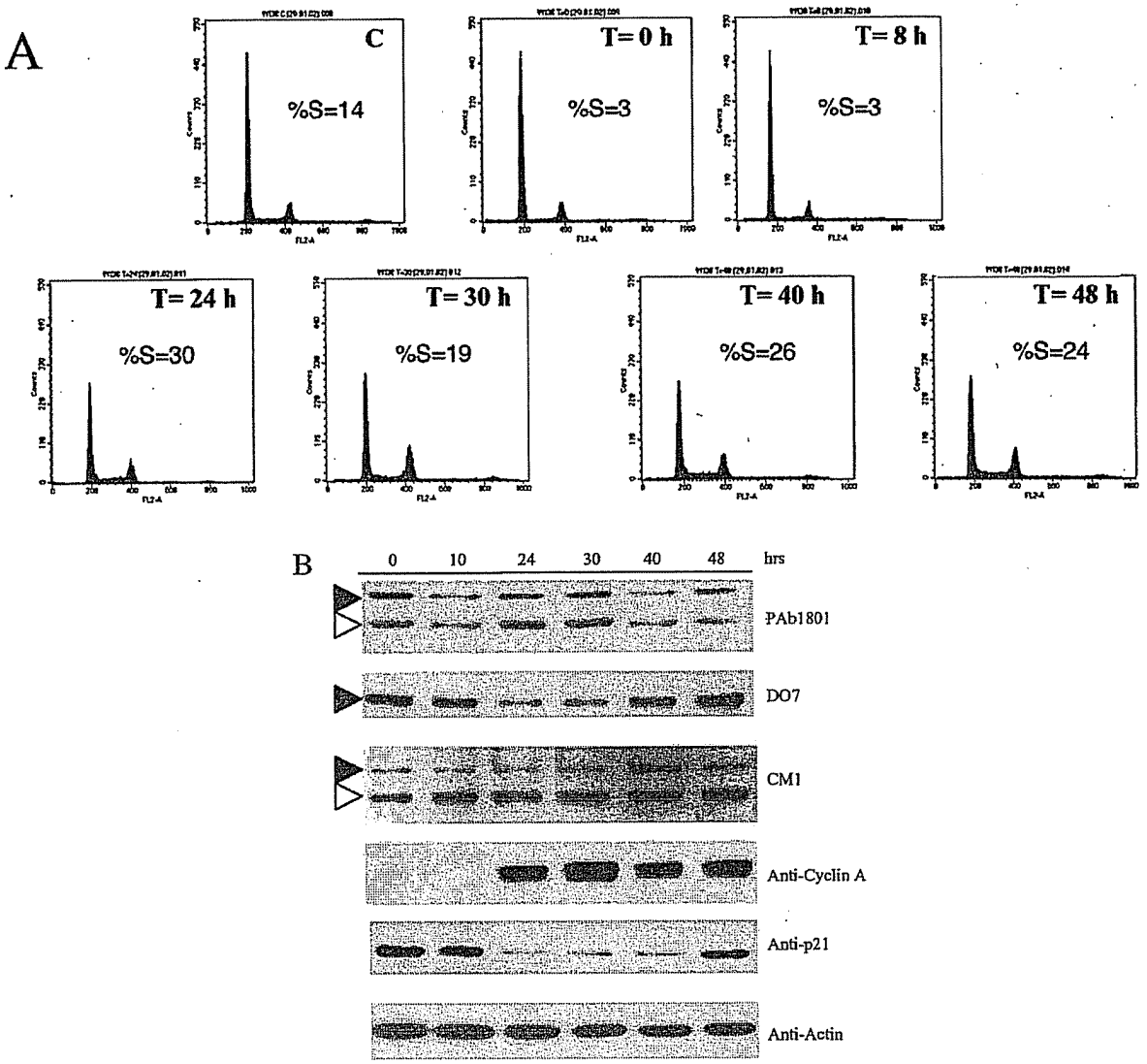

Figure 4 Variation of FL-p53/ΔN-p53 ratio during cell cycle progression in WI-38 human diploid fibroblasts. WI-38 fibroblasts, which spontaneously express both FL-p53 and ΔN-p53 were arrested in Go by serum starvation for 72 h and then restimulated with serum to trigger synchronous cell-cycle progression. At various time points after restimulation, cells were harvested and analysed for cell-cycle distribution by flow cytometry after staining with propidium iodide (cycle TEST^{TM} Plus Kit, Becton Dickinson), and analysed by Western blot using PAb1801, DO7, CM1, anti-Cyclin A (Sigma), anti-P21^{WAF1} (Santa Cruz) and anti-β actin (Santa Cruz) as a loading control. Black arrow: FL-p53; white arrow: ΔN-p53

whether ΔN-p53 is required for cell-cycle progression in cells expressing wild-type p53.

There is a clear structural homology between ΔN-p53 and the ΔN-isoforms of p63 and p73. Both of these isoforms are characterized by a lack of N-terminal transactivation domains. As for ΔN-p53, ΔN-p63 and ΔN-p73 appears to behave as negative regulators of their full-length counterparts. However, in contrast with ΔN-p53, the truncated N-terminal p63 and p73 proteins were derived from alternative splicing as well as, in the case of p63, an alternative promoter and initiation codon in intron 3 (Levrero *et al.*, 2000; Yang *et al.*, 1998, 2002). Interestingly, p63 and p73 are mainly regulated at the transcriptional level, whereas p53 is essentially controlled by translational and post-translational mechanisms (enhanced mRNA translation (Reich *et al.*, 1983) and control of protein stability (Haupt *et al.*, 1997; Kubbutat *et al.*, 1997)). This dichotomy is apparently also reflected in the mode of generation of the respective ΔN-isoforms. We thus propose that the existence of ΔN-isoforms, deleted for their main transcriptional activation domain, is a characteristic of all members of the *TP53* gene family. A ΔN-isoform of p53 identical to the one reported here, termed p53/47, was recently described by Yin *et al.* (2002). Consistent with our results, this p53/47 was resistant to Mdm2-mediated proteosomal degradation. However, Yin *et al.* (2002) reported that p53/47 had an autonomous pro-apoptotic activity, an observation not supported by our data (Figure 3f). Given the potential

of these ΔN-isoforms to act either as autonomous factors or 'anti-suppressors' of FL-p53, their involvement in the regulation of cell growth and apoptosis deserves further investigations.

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